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A critical step in tumorigenesis is proteolytic modification of the peri-cellular matrix surrounding tumor cells by matrix metalloproteinases (MMPs). Stromal cells associated with tumors, not the tumor cells themselves, are responsible for the production of most tumor MMPs. Studies from our laboratory and those of our collaborators have shown that EMMPRIN (extracellular matrix metalloproteinase inducer), a tumor cell surface glycoprotein, stimulates the production of several MMPs by fibroblasts and endothelial cells. Antisense cDNA and ribozyme constructs were utilized in an attempt to inhibit EMMPRIN expression in TA3/ST cells, a murine breast carcinoma cell line. These constructs were not efficient in blocking EMMPRIN expression and consequently, were inactive *in vivo*. However, transfection and injection experiments done in collaboration with Dr. Stanley Zucker have shown that MDA-MB-436 human breast cancer cells transfected with GFP-EMMPRIN can produce much larger tumors in nude mice than vector-transfected cells. Also, EMMPRIN can stimulate the production of MMPs 1, 2 and 3 by endothelial cells; MMPs 1, 2 and 9 have been shown previously to promote angiogenesis. Therefore, we proposed that a possible explanation of the increased tumor growth obtained with EMMPRIN-transfected cells is an efficient nutrient supply resulting from angiogenesis. To assay whether EMMPRIN is capable of inducing angiogenesis, we treated HUVECs on type I collagen with either EMMPRIN or bFGF, a known angiogenic factor. As opposed to controls which maintained their cobblestone-like monolayer arrangement, treated HUVECs formed capillary-like tubules, lending support to EMMPRIN as an angiogenic factor.

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FOREWORD

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Introduction

A critical step in tumorigenesis is proteolytic modification of the peri-cellular matrix surrounding tumor cells by matrix metalloproteinases (MMPs). Stromal cells associated with tumors, not the tumor cells themselves, are responsible for the production of most tumor MMPs. Studies from our laboratory and those of our collaborators have shown that EMMPRIN (extracellular matrix metalloproteinase inducer), a tumor cell surface glycoprotein, stimulates the production of several MMPs by fibroblasts and endothelial cells. Antisense cDNA and ribozyme constructs were utilized in an attempt to inhibit EMMPRIN expression in TA3/ST cells, a murine breast carcinoma cell line. These constructs were not efficient in blocking EMMPRIN expression and consequently, were inactive *in vivo*. However, transfection and injection experiments done in collaboration with Dr. Stanley Zucker have shown that MDA-MB-436 human breast cancer cells transfected with GFP-EMMPRIN can produce much larger tumors in nude mice than vector-transfected cells. Also, EMMPRIN can stimulate the production of MMPs 1, 2 and 3 by endothelial cells; MMPs 1, 2 and 9 have been shown previously to promote angiogenesis. Therefore, we proposed that a possible explanation of the increased tumor growth obtained with EMMPRIN-transfected cells is an efficient nutrient supply resulting from angiogenesis. To assay whether EMMPRIN is capable of inducing angiogenesis, we treated HUVECs on type I collagen with either EMMPRIN or bFGF, a known angiogenic factor. As opposed to controls which maintained their cobblestone-like monolayer arrangement, treated HUVECs formed capillary-like tubules, lending support to EMMPRIN as an angiogenic factor.

Body

Task 1: To show that inhibition of EMMPRIN protein expression leads to a reduction in breast tumor growth.

In an effort to show that inhibition of EMMPRIN protein expression leads to a reduction in breast tumor growth and metastasis, we constructed both human and murine EMMPRIN antisense cDNAs and ribozymes. These constructs were then transfected into TA3/ST murine breast carcinoma cells or human MDA-MB-231 human breast carcinoma cells. The stably transfected cells were then injected into the tail vein of syngeneic mice to see if growth and metastasis of the tumor would now be reduced. Neither of the constructs was efficient in blocking EMMPRIN expression, and consequently were inactive *in vivo*. This effect is currently under further investigation by other members of the laboratory.

Task 2: To demonstrate a rise in MMP production by microvascular endothelial cells as well as umbilical vein endothelial cells in response to EMMPRIN treatment.

Tumor angiogenesis plays an important role in cancer progression (1). Since up-regulation of MMP activity is required for angiogenesis and tumor endothelial cells express MMPs (2,3), Dr. Stanley Zucker, in collaboration with my mentor's laboratory examined whether EMMPRIN stimulates MMP production by human endothelial cells, as well as fibroblasts. In three separate experiments, purified EMMPRIN was shown to cause a significant stimulation of stromelysin-1, gelatinase A and collagenase (MMP-1) production (but not TIMP-1 or TIMP-2) in human umbilical vein endothelial cells. The effect was most marked for stromelysin since the background levels produced by the untreated endothelial cells were low. In contrast, VEGF, an established mediator of angiogenesis, had little effect on stromelysin or gelatinase A and a relatively strong effect on MMP-1; but, the most dramatic effect of VEGF was on TIMP-1 stimulation (4). These effects were not due to influences on proliferation. Thus, EMMPRIN is a more potent inducer of MMP synthesis than VEGF.

Further evidence for a role for EMMPRIN in tumor angiogenesis has developed out of the collaboration between Dr. Zucker's and my mentor's laboratories. A line of MDA-MB-436 human breast cancer cells that grows slowly *in vivo* was transfected with EMMPRIN cDNA or with vector alone, and tested for tumor growth and metastasis in nude mice. Whereas control vector transfectants formed very small, non-metastatic tumors, the EMMPRIN transfectants in most cases formed large palpable tumors and metastasized to numerous sites. The latter tumors, but not the former, were found to be highly vascularized.

My major role in these projects has been to develop a better system for demonstrating

EMMPRIN-dependent MMP stimulation in endothelial cells. I am currently examining MMP production by human umbilical vein endothelial cells that have been infected with a recombinant, full-length EMMPRIN adenovirus. This approach is being utilized due to the availability of adenoviral reagents in the laboratory as well as ease of amplification and purification. In the past, we have used EMMPRIN purified from LX-1 cells, a human lung carcinoma cell line. The purification process is tedious, time-consuming, labor-intensive and yields small quantities of EMMPRIN. Utilizing the adenoviral approach will not only provide us with another way to generate EMMPRIN, but will also eliminate possible contaminants that may be in our EMMPRIN preparations purified from LX-1 cells. This approach has been shown to provide more reproducible and efficient stimulation of MMP production by fibroblasts.

In our adenoviral approach, human umbilical vein endothelial cells are infected with the EMMPRIN adenovirus. We expect that EMMPRIN will be produced by the endothelial cells and bind to its putative receptor on neighboring endothelial cells, leading to a series of signaling events that will ultimately result in MMP production. This approach is now underway.

Task 3: To demonstrate endothelial cell invasion and tubule formation induced by EMMPRIN, or an augmentation of VEGF-induced endothelial cell invasion and tubule formation by EMMPRIN.

Task 3 has been my major project over the past year.

One of the systems commonly used to mimic aspects of angiogenesis involves culturing of endothelial cells on 3-dimensional gels of type I collagen or Matrigel. Under appropriate stimulus, the endothelial cells invade the gel and form capillary-like, tubular structures. In one such study (1), human umbilical vein or human dermal microvascular endothelial cells were seeded on collagen gels and treated with a phobol ester (PMA). With both cell types, PMA treatment induced invasion of the gels and formation of vessel-like structures within the gels, along with increased levels of MMP-1 and gelatinase A. Inhibition of MMP activity especially MMP-1, prevented the induction of endothelial cell invasion and morphogenesis by PMA. These experiments imply that stimulation of MMP activity is necessary, and possibly sufficient, for induction of invasion and tubule formation in this system.

We have conducted experiments in which human umbilical vein endothelial cells were cultured in 12-well plates ($5X10^{5}$) on a type I collagen gel and treated with either bFGF, a known angiogenic agent, or purified EMMPRIN, a known MMP stimulator. Untreated cells maintain a cobblestone-like pattern, whereas both bFGF- and EMMPRIN-treated endothelial cells form capillary-like tubules, as visualized by phase constrast microscopy. Tubule formation is such, that treating cells with 1ug of purified EMMPRIN yields visual results comparable to those obtained with 5ng of bFGF. We believe that the high ammount of EMMPRIN needed is due to inactivation of most of the protein during purification.

Due the the constraints in purifying EMMPRIN from LX-1 cells that were mentioned above in Task 2, we are now utilizing an adenoviral approach. The human umbilical vein endothelial cells are cultured in 12-well plates on type I collagen. Rather than adding exogenous EMMPRIN, we infect the cells with a recombinant, full-length EMMPRIN adenovirus, enabling the cells to make EMMPRIN themselves. By the same principle mentioned previously, we expect the EMMPRIN on the surface of a given endothelial cell to interact with its putative receptor on a neighboring endothelial cell, triggering a series of signaling events that will result in tubule formation, presumably by MMP stimulation. These cultures are assayed by phase contrast microscopy, Western analysis and ELISA.

Key Research Accomplishments

- Construction of EMMPRIN antisense cDNAs and ribozymes.
- Stable transfection of TA3/ST murine breast carcinoma cells and MDA-MB-231 human breast carcinoma cells with the previously mentioned constructs.
- EMMPRIN-stimulated capillary-like tubule formation by human umbilical vein endothelial cells on type I collagen.
- Initiation of EMMPRIN-dependent MMP stimulation and tubule formation experiments with human umbilical vein endothelial cells utilizing a recombinant, full-length EMMPRIN adenovirus.

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